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Detection of trace amounts of hidden allergens: hazelnut and almond proteins in chocolate

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Abstract

Many patients with immediate type allergy to tree pollen also suffer from intolerance to hazelnuts and almonds. Since rather low levels of hazelnut and almond proteins can provoke an allergic reaction in sensitized individuals, an immunoblot technique has been developed for the detection of potentially allergenic hazelnut and almond proteins in chocolate. Initially, IgE binding hazelnut and almond proteins were detected by immunoprobing with allergic patients' sera. For routine analysis, patients' sera were substituted with polyclonal rabbit antisera, and sensitivity was enhanced by the use of a chemiluminescent detection method. This technique allowed the detection of less than 0.5 mg of hazelnut or almond proteins per 100 g of chocolate (=5 ppm). It was applied for routine screening purposes in product quality control as well as for optimization of cleaning steps of filling facilities to minimize cross contamination during production.

Keywords: Food allergy; Immunoblotting; Almond; Hazelnut; Proteins

1. Introduction

In the developed countries of the northern hemisphere, allergic diseases of the upper respiratory system (e.g. pollinosis) are quite frequent, affecting up to 10% of the population. Many of the atopic patients additionally suffer from food-related allergies, often due to cross-reactivites between pollen (birch, hazel) and food allergens [1]. The prevalence of food allergy has been estimated up to 1% among adults and 2–3% among children in these countries [2,3].

'True' food allergies are immediate type allergic reactions mediated by allergen-specific IgE antibodies. The vast majority of food allergens are proteins. Besides animal products (milk, egg, fish), vegetable foods such as legume seeds, tree nuts and fruits are important sources of allergens. While fruits (e.g. apples) mainly cause oral symptoms, legume seeds and nuts also induce acute generalized symptoms and even anaphylactic shock [4,5].

Food products often contain non-declared ingredients. The reason for this varies from illegal substitution for higher priced components to unintended (cross-)contamination during processing. Unexpected

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contact with milligram quantities of these ingredients can cause severe allergic reactions in people with hypersensitivity to these particular food components [6].

In contrast to inhalant allergies, food allergies cannot be treated successfully by specific desensitization. Up to now, the only treatment is avoidance of the causative agent. Consequently, it is of importance for food allergic persons to be able to obtain food products which are free of certain allergenic ingredients.

Although there are no generally accepted limits for allergenic compounds in processed food, the World Health Organization's (WHO) recommendations for gluten-free products (less than 1 mg of gluten/100 g dry product) may serve as a guideline [7]. As a consequence, analytical methods with a high detection capability are mandatory to certify the allergen content of a food product.

Due to the large number of birch and hazel pollen allergic subjects in Northern Europe, chocolates produced for the Scandinavian market ought to be free of hazelnut and almond proteins. Several methods have been reported for the detection of protein contaminants in food. The most popular of them are antibody based techniques such as immunodiffusion or enzyme-linked immunosorbent assay (ELISA). Both methods, although being useful for food analysis in many cases, suffer from several drawbacks when it comes to the analysis of trace amounts of hazelnut and almond proteins in chocolate. Unfortunately, analytical techniques based upon immunodiffusion are usually not sensitive enough to detect these proteins in the lower ppm range, whereas with ELISA procedures, which are more sensitive than immunodiffusion assays, the food matrix (i.e. chocolate) tends to interfere with detection. Until recently, no ELISA-based techniques sensitive enough to detect trace amounts of hazelnut and almond proteins in chocolate were available (see also Addendum).

In order to avoid interference by matrix proteins, in the present study a combination of electrophoretic separation followed by a highly sensitive immunochemical detection method has been applied. Initially, the major IgE binding hazelnut and almond proteins were detected by immunoblotting with allergic patients' sera. For routine analysis, patients' sera were substituted with polyclonal animal sera, and sensitivity was enhanced by the use of a highly-sensitive chemiluminescent detection method. Finally, the optimized immunoblotting technique was applied in quality control for the detection of trace amounts of hazelnut and almond proteins, and to minimize cross-contamination during the production of chocolate.

2. Experimental

2.1. Chemicals

Dithiothreitol (DTT), Tris(hydroxymethyl)-aminomethane (Tris), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), Nitroblue tetrazolium (NBT), polyvinylpyrrolidone (PVP, M_r 40 000), alkaline phosphatase-labelled goat-anti human IgE and anti rabbit IgG were from Sigma (St. Louis, MO, USA). Acrylamide (2×cryst.), *N,N′*-methylenebisacrylamide and sodium dodecyl sulfate (SDS) were from Serva (Heidelberg, Germany). All other chemicals for electrophoresis and staining were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical or biochemical grade. Western-Light™ Chemiluminescent Detection System was from Tropix (Bedford, MA, USA). GelBond PAGfilm^{R} was from Amersham Pharmacia (Uppsala, Sweden). PVDF membrane (Immobilon P, pore size 0.45 μm) was from Millipore (Bedford, MA, USA). Fount India Ink was from Pelikan (Hannover, Germany). Reagents for the determination of specific IgE (CAP-Test) were from Pharmacia (Uppsala, Sweden). Reagents for skin prick test were from Allergopharma (Reinbeck, Germany).

2.2. Sera

2.2.1. Patient sera

Sera were obtained from 12 patients suffering from nut and/or tree pollen allergy of the immediate type. A diagnostic procedure (typical case history, positive skin prick test, specific IgE antibodies in the serum of CAP class 3–6) confirmed that these persons were allergic to hazel pollen, hazelnut and/or almond allergens.

2.2.2. Polyclonal antisera

Rabbit polyclonal antisera against hazelnut and almond proteins were obtained from Dako (Hamburg, Germany) and Behring (Marburg, Germany), respectively. The specifity of antisera has been checked with a dot-immunobinding assay (dot-blot). With this assay, no cross-reactions between hazelnut/almond-proteins and milk-, cocoa-, or peanut-proteins were detectable.

All sera were stored at −20°C until used.

2.3. Equipment

All apparatus for horizontal electrophoresis (Multiphor II electrophoresis chamber, Macrodrive I power supply) and blotting (Novablot, Film-Remover) were from Amersham Pharmacia (Uppsala, Sweden). For scanning immunostained PVDF membranes or films, a Molecular dynamics 300A laser scanning densitometer (Sunnyvale, CA, USA) was used.

2.4. Sample Preparation

2.4.1. Extraction of hazelnuts and almonds

Raw and roasted hazelnuts and almonds were ground with a mortar and a pestle until they passed a 0.1 mm sieve. For protein extraction, the flour (100 mg) was mixed with 5 ml of extraction buffer (25 m*M* Tris–HCl pH 8.8, 2% SDS, 0.5% DTT, 10% glycerol, 0.005% Bromophenol blue dye) and stirred for 45 min at 20°C. After centrifugation at 16 000 *g* for 10 min, the supernatant was heated in a boiling water bath for 5 min, cooled down to 50°C, and after adding 0.2% DTT, stored at −20°C for further use.

2.4.2. Extraction of chocolate samples

Chocolates (83 different samples) which had been produced for the Scandinavian Market and which were supposed to be free of hazelnuts and almonds were obtained from different suppliers. Chocolates with defined admixtures (0.005–5%) of hazelnuts or almonds were prepared in our lab. All samples were frozen (−20°C) to facilitate grinding with a mortar and pestle. Representative samples (1.0 g) were mixed with 25 ml of extraction buffer and extracted as described above for hazelnut and almond proteins.

2.5. SDS-Electrophoresis

SDS-Electrophoresis was performed on a horizontal system using 0.5 mm thin gels [8] on GelBond PAGfilm^{R}. Discontinuous SDS gels (250×125 mm^{2}) with a T=4.5% stacking gel and a T=12.5% resolving gel (C=3%, 0.1% SDS, 375 m*M* Tris–HCl pH 8.8) were cast as described previously [9]. Electrode buffer was 25 m*M* glycine, 192 m*M* Tris and 0.1% SDS [10]. Proteins were stacked at 150 V and resolved at 500 V at 15°C. Sample load was 10 μl per lane.

2.6. Electrophoretic transfer (blotting)

The electrophoretically separated proteins were electroblotted onto an immobilizing PVDF membrane. The blotting procedure was based on a modification of the system described by Kyhse-Andersen [11]. Blotting buffer was 25 m*M* glycine, 192 m*M* Tris, 0.1% SDS and 20% methanol. Transfer was performed at 0.8 mA cm^{-2} for 60 min at room temperature. After termination of the electrophoretic transfer, the PVDF membrane was washed 2×(5 min each) with TBS (25 m*M* Tris–HCl pH 7.4 and 200 m*M* NaCl) and air-dried. To check whether the electrophoretic transfer had been performed correctly, a piece of membrane (3 cm wide) was cut off at one edge and stained with the general protein stain Indian Ink [12].

2.7. Immunodetection of hazelnut and almond allergens/antigens

The dried PVDF membrane was wetted in methanol and washed twice with TBST (25 m*M* Tris–HCl pH 7.4, 200 m*M* NaCl, 0.1% Tween 20). To saturate unoccupied binding sites, the membrane was incubated with blocking buffer (2% PVP+1% BSA dissolved in TBST) for 3 h at room temperature.

The detection of IgE binding proteins (allergens) was performed as described previously [13,14]. Briefly, PVDF membrane was incubated overnight with the sera of allergic individuals (dilution: 1:20 in TBST+1% BSA). After three washes (5 min each) with TBST, the membrane was then incubated for 6 h with alkaline phosphatase-labelled goat anti-human IgE (dilution: 1:2000 in TBST+1% BSA). After two

additional washes (5 min each) with TBST, the bound allergens were visualized with BCIP/NBT staining [15].

For the detection of antigens, the membrane was incubated for 2 h with rabbit antisera against hazelnut or almond proteins (serum dilution: 1:2000 in TBST+1% BSA). Following three washes (5 min each) with TBST, the membrane was incubated for 2 h with alkaline phosphatase-labelled goat anti-rabbit IgG (dilution: 1:30 000 in TBST+1% BSA). After two additional washes (5 min each) with TBST, the bound antigens were visualized with BCIP/NBT staining. Alternatively, hazelnut and almond antigens were visualized with the Western-Light™ Chemiluminescent Detection System according to the manufacturer's instructions.

2.8. Densitometric evaluation

The stained blots or films were digitized at 88 μm resolution. The resulting images were analyzed using the QUANTITY ONE software package (PDI, NY, USA).

3. Results and discussion

3.1. Identification of IgE-binding hazelnut and almond proteins

In order to identify the major hazelnut and almond allergens, hazelnut and almond proteins were separated by SDS–PAGE, transferred onto PVDF membrane and immunoprobed with patients' sera. Applying this technique, serum IgE from 12 persons displaying type I allergic reactions to tree pollen and/or sensitization to hazelnuts or almonds bound to hazelnut proteins in the M_r-range between 14 and 90 kDa. The serum from each of the 12 allergic individuals exhibited a unique IgE-binding pattern. Major IgE binding occurred with hazelnut proteins in the 18, 25, 32, 38, 49, and 65 kDa range. IgE binding to almond proteins was less pronounced than with hazelnut; IgE binding almond proteins were located in the 20, 22, 25, 48, and 65 kDa region (results not shown).

The same IgE-binding proteins were detectable in chocolate containing an admixture of 5% hazelnuts and almonds, respectively (Fig. 1). In a control experiment, almost no IgE-binding was observed with the serum of an individual allergic to grass pollen (Fig. 1).

3.2. Detection of trace amounts of hazelnut and almond proteins in chocolate by immunoprobing with rabbit polyclonal antiserum

Although it may be possible to detect low amounts of hazelnut and almond proteins in chocolate by immunoblotting with allergic persons' sera, for routine analysis purposes in food processing industry, however, the use of patients' sera is excluded because of several reasons, e.g. limited availability and quality of sera, health hazard etc. Therefore, in the following experiments, patients' sera were substituted with polyclonal antisera raised in animals.

3.2.1. Specifity of antisera

Commercially available antisera against hazelnut and almond proteins were used in the following experiments. The specifity of these antisera was analyzed by immunoblotting. Due to the close phylogenetic relationship of almonds and hazelnuts, the antiserum raised against hazelnut proteins also reacted with a number of almond proteins and vice versa (Fig. 2, Lanes 1 and 2). However, almond could be distinguished from hazelnut by the size distribution pattern of the protein bands recognized by these antisera.

Both antisera did neither react with milk proteins (which are a common ingredient of chocolates) (Fig. 2, Lane 3) nor with cocoa proteins. A 5% admixture of hazelnuts and almonds to chocolate was easily detectable (Fig. 2, Lanes 4 and 5).

3.2.2. Detection limit of hazelnut and almond proteins in chocolate

Chocolates with known concentrations (0.005–5%) of hazelnuts or almonds were analyzed by immunoblotting with rabbit antisera and secondary, alkaline phosphatase labelled antibody and BCIP/NBT as substrate/chromogen.

The immunoblots presented in Fig. 3 indicate major hazelnut antigen bands in the M_r range of 17, 20, 26, 30, 38, 49 and 66 kDa (Fig. 3a). The 38 and 49 kDa bands, which had been identified as allergens in Section 3.1, were detectable down to a concentration of 0.005% hazelnut in chocolate. Since

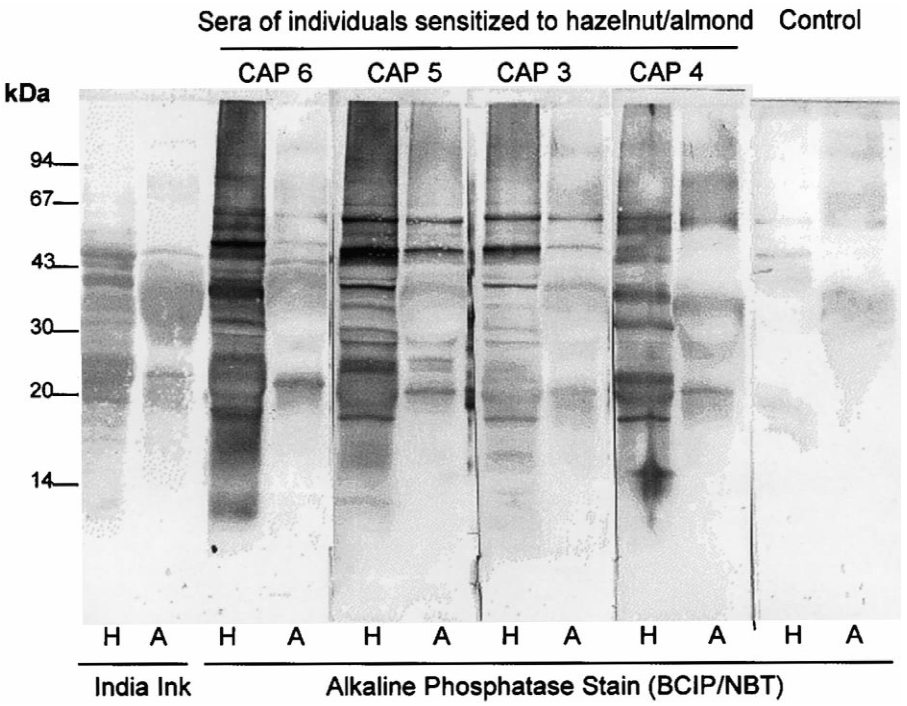

Fig. 1. Detection of IgE-binding hazelnut and almond proteins. SDS–PAGE and immunoblotting with sera of individuals allergic to tree pollen and hazelnuts (CAP classes 3–6). *Left:* Total protein stain with Indian Ink. *Center:* Immunostain with patients' sera. *Right:* Immunostain with a serum of an individual allergic to grass pollen (control experiment). H = hazelnut, A = almond.

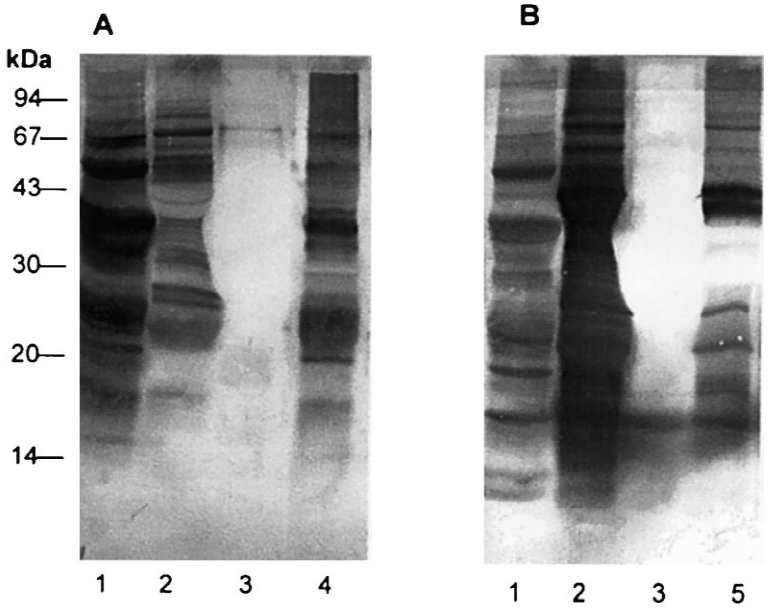

Fig. 2. Specifity of rabbit polyclonal antisera directed against hazelnut and almond proteins. SDS–PAGE and immunoblot. (A) antiserum directed against hazelnut proteins. (B) = antiserum directed against almond proteins. 1 = hazelnut extract; 2 = almond extract; 3 = Milk chocolate; 4 = hazelnut chocolate; 5 = almond chocolate.

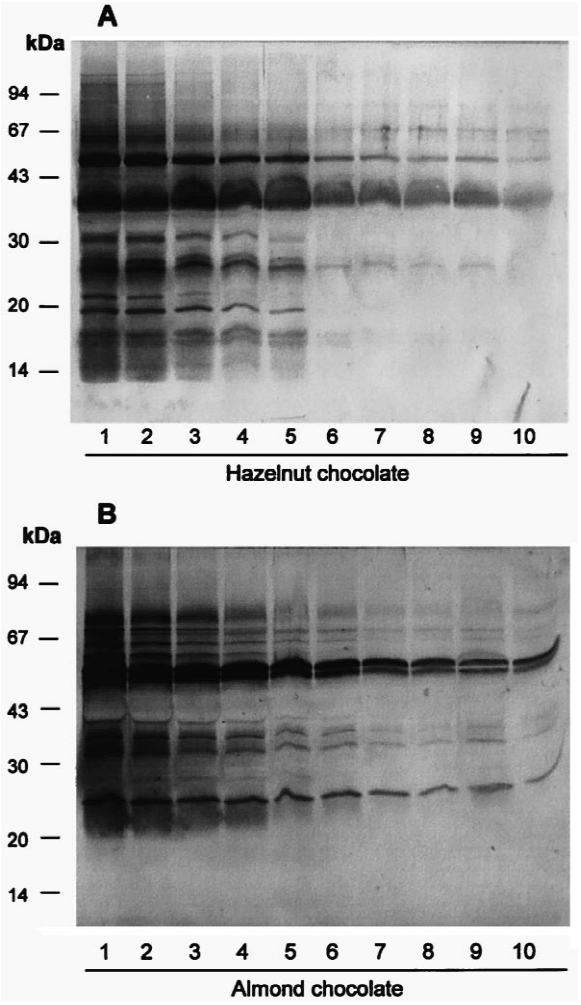

Fig. 3. Detection limit of hazelnut and almond proteins in chocolate. BCIP/NBT stain. SDS–PAGE and immunoblot with rabbit antiserum against (A) hazelnut and (B) almond proteins. (1–10) = chocolates with definded concentrations of (A) hazelnuts and (B) almonds: 1 = 5%, 2 = 2.5%, 3 = 1%, 4 = 0.5%, 5 = 0.25%, 6 = 0.1%, 7 = 0.05%, 8 = 0.025%, 9 = 0.01%, 10 = 0.005%.

hazelnut contains roughly 10% protein, the detection limit for hazelnut protein is even one order of magnitude lower (0.5 mg/100 g of chocolate).

In almonds, the picture is similar (Fig. 3b): Two strong 46 and 48 kDa bands, and a lesser reactive 25 kDa band are detectable down to 0.005% almonds in chocolate, which equals 0.5 mg almond protein/100 g of chocolate.

In order to enhance sensitivity of the detection step, BCIP/NBT was subsituted by the more sensitive chemiluminescent substrate CSPD^{R} (Western-Light™). Using this enhanced chemiluminescent (ECL) technique, 0.5 mg of hazelnut or almond proteins/100 g of chocolate could be detected with certainty (Fig. 4) which means that sensitivity is high enough to fulfill the WHO recommendations (cf. Introduction section).

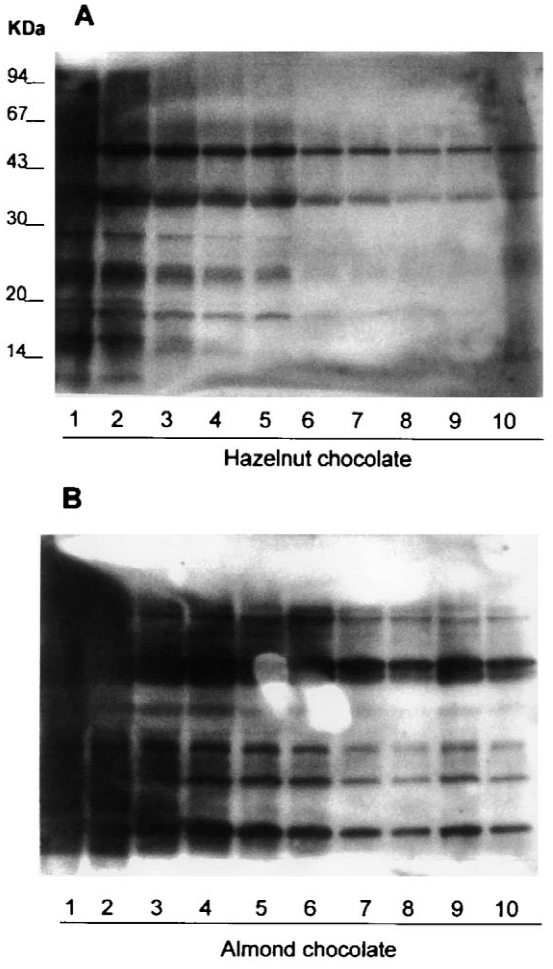

Fig. 4. Detection limit of hazelnut and almond proteins in chocolate. Detection with enhanced chemiluminescence (Western Light™). SDS–PAGE and immunoblot with rabbit antiserum against (A) hazelnut and (B) almond proteins. (1–10) = chocolates with definded concentrations of (A) hazelnuts and (B) almonds. Concentrations as in Fig. 3.

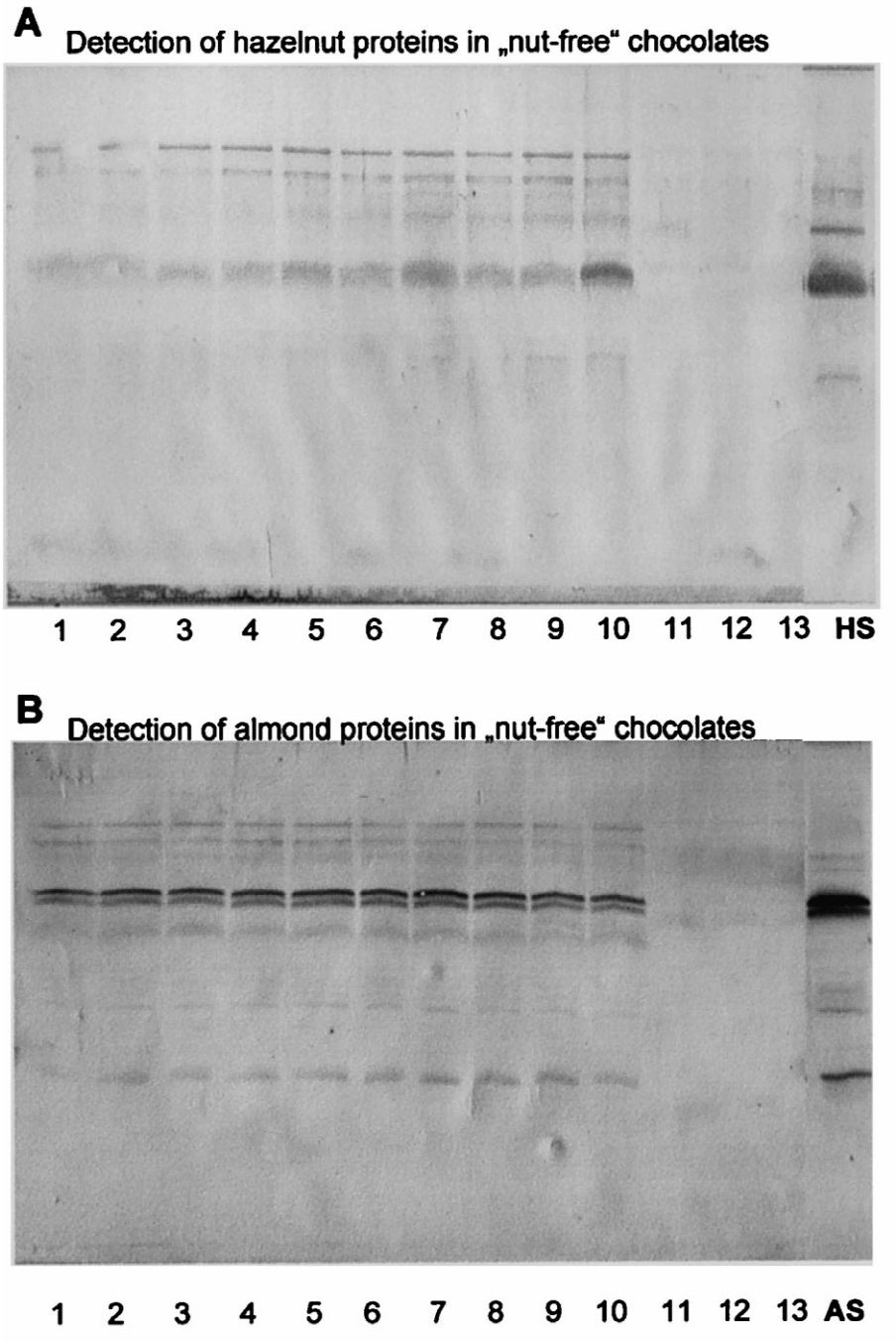

Fig. 5. Screening of chocolates for traces of hazelnut and almond proteins. SDS–PAGE and immunoblot with rabbit antiserum against (A) hazelnut and (B) almond proteins. (1–13) = chocolates produced for the Scandinavian market from different suppliers. HS = hazelnut, AS = almond standards (0.002% hazelnut/almond protein) for comparison.

3.3. Screening of chocolates for traces of hazelnut and almond proteins

The immunoblotting technique was also applied for screening different chocolates which had been produced for the Scandinavian market and which were supposed to be free of hazelnut and almond proteins. In total, 83 samples were analyzed. Surprisingly, in 60 samples the concentration of hazelnut proteins exceeded 1 mg per 100 g; almond protein concentrations exceeding 1 mg per 100 g were found in 51 samples (see Fig. 5a and b).

3.4. Optimization of cleaning steps to minimize cross contamination

Because of its low prize, nougat is a common ingredient in chocolates. On the other hand, due to its widespread use, it is a major source of cross contamination of chocolates with hazelnut proteins. In order to minimize carry over during processing, the filling facilities have to be thoroughly cleaned prior to filling nut-free chocolate.

The immunoblotting technique was applied to study and improve the efficiency of different cleaning steps. A typical example is given in Fig. 6, where the efficiency of cocoa butter for cleaning the filling machine was investigated.

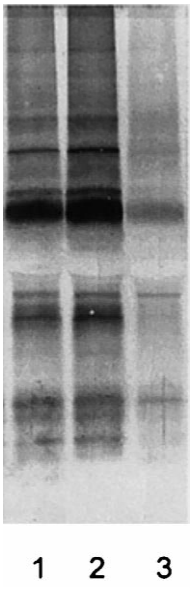

Fig. 6. Carry over of hazelnut proteins and efficiency of cleaning steps. Detection of hazelnut proteins in chocolate (1) after having filled hazelnut chocolate and cleaned the filling machine with cocoa butter, (2) after having filled hazelnut chocolate without cleaning with cocoa butter and (3) after having filled milk chocolate and cleaned with cocoa butter. SDS–PAGE and immunoblot with rabbit antiserum against hazelnut proteins.

3.5. Conclusions

An immunoblot technique using rabbit antisera and chemiluminescent detection has been developed for the detection of low levels of potentially allergenic hazelnut and almond proteins in chocolate. The method allows the detection of less than 0.5 mg of hazelnut or almond proteins per 100 g of chocolate. It is applicable for routine screening purposes in product quality control as well as for optimization of cleaning steps of filling facilities to minimize cross contamination during production.

4. Addendum

After submission of this manuscript, three papers on the detection of trace amounts of hazelnuts and almonds in foods by ELISA were published (see Refs. [16–18]).

5. Nomenclature

% C	= cross-linking agent (g/100 ml)/%T
%T	= total acrylamide plus *N,N′*-methylenebisacrylamide concentration (g/100 ml)
BCIP	= 5-bromo-4-chloro-3-indolyl phosphate
BSA	= bovine serum albumin
DTT	= dithiothreithol
ECL	= enhanced chemiluminescence
ELISA	= enzyme-linked immunosorbent assay
IgE	= immunoglobulin E
NBT	= Nitroblue tetrazolium
PAGE	= polyacrylamide gel electrophoresis
PVDF	= polyvinylidene difluoride
PVP	= polyvinylpyrrolidone
SDS	= sodium dodecyl sulfate
TBS	= Tris buffered saline
TBST	= Tris buffered saline + Tween 20
Tris	= Tris(hydroxymethyl)aminomethane

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